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TNT Detection Using Multiplexed Liquid-Array Displacement Immunoassays

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Introduction: The presence of trace environmental contamination of soil and ground water is an ongoing concern. Historically, improper practices during the manufacture and storage of explosives, such as 2,4,6-trinitrotoluene (TNT), were the primary causes. Now, the need for trace detection of explosives is further heightened by current homeland security concerns. Required are easy-to-use, sensitive, and fast methods to facilitate the detection and quantification of these contaminants, whether to monitor remediation or provide surveillance. Biosensors have been found effective for solution-phase detection of environmental contaminants, with NRL pioneering the use of flow displacement immunoassays.^{1,2} Our work utilized the Luminex¹⁰⁰ (a commercial flow cytometer), which can perform highly multiplexed assays by discriminating between up to 100 different bead sets, each of which can be used to test for a different target. While the Luminex¹⁰⁰ has been shown to be a highly effective biosensor for sandwich or competitive immunoassays, the goal of this project was to demonstrate for the first time a displacement immunoassay on this platform, and to test its suitability for TNT detection in the marine environment.

We evaluated four different TNT monoclonal antibodies, two recombinant TNT antibodies, and a control antibody simultaneously for the rapid detection of TNT and other nitroaromatics. TNT was detected at 0.1 ppb and could be quantified over the range of 1 ppb to 10 ppm. In addition, the assay was shown to be effective in various matrixes such as lake water, sea water, and soil extracts. This ability to function in the maritime environment is critical for Naval applications, such as trace detection of explosive materials onboard interdicted vessels, anti-mine operations, or detection of unexploded ordinance. In addition, the assay methodology can be applied to address the whole scope of small-molecule detection needs, from environmental pollutants to identification of illicit drugs.

TNT Displacement Immunoassay: The Luminex¹⁰⁰ is a specialized flow cytometer that performs multiplexed immunoassays by using a red laser to differentiate up to 100 different types of fluorescent latex microspheres, while a green laser quantifies a shorter wavelength fluorophore or fluorescent target molecule bound to the surface of each microsphere

during the assay. Luminex microspheres (seven types, each coated with a different antibody) were incubated with a high concentration of the fluorescent analog of TNT, Alexa Fluor 555-TNB (AF-TNB). Afterwards, the excess AF-TNB was removed, the beads added to the samples, and immediately processed in the instrument to analyze for TNT. If TNT was present, AF-TNB was displaced in a TNT concentration dependent manner from the bead surface (Fig. 10), resulting in a lower measured signal on each bead. By measuring the loss in signal on each bead set and plotting median fluorescent intensity vs TNT concentration, we could quantify the concentration of TNT over the range of 10 ppm to 1 ppb (Fig. 11). The limit of detection for TNT varied for the different antibodies tested, with Mab A1.1.1 being the most sensitive at 0.1 ppb.

The relative cross-reactivities of all the TNT antibodies to a panel of nitroaromatic or nitramine compounds were also determined. In this displacement assay, the cross-reactivity factor was calculated by first determining the concentration of each compound required to produce 50% displacement of the AF-TNB from the beads. This concentration was compared to the amount of TNT required to induce 50% displacement (Table 1). The magnitude is indicative of how much more of each compound is required to generate the same displacement of AF-TNB as TNT. For the monoclonal antibodies, tetaryl was found to be more effective, while unrelated compounds like RDX gave essentially no cross-reactivity.

Tests were also performed on acetone extracts of soil samples collected from areas contaminated with TNT. Table 2 shows the results of these analyses and compares them to analyses of these same acetone extracts by the EPA standard high-performance liquid chromatography (HPLC) method.

Summary: We demonstrated the potential of the multiplexed fluid-array displacement immunoassay to detect the explosive TNT and related compounds. This assay was highly sensitive with a large dynamic range. The test was rapid, taking only a few minutes to complete, and user-friendly, requiring the addition of only a single reagent prior to measurement. This method was found very useful for choosing the best anti-TNT antibody, and also made possible the rapid comparison of their cross-reactivities for various explosive compounds. By using antibodies with complementary cross-reactivities and a pattern recognition algorithm, both the identity and concentration of the explosive could potentially be determined. The next step is to demonstrate that this assay can monitor a range of unrelated explosive targets, using one or more antibody-coated microspheres and suitable fluorescent analogs for each target to be detected. This ability to

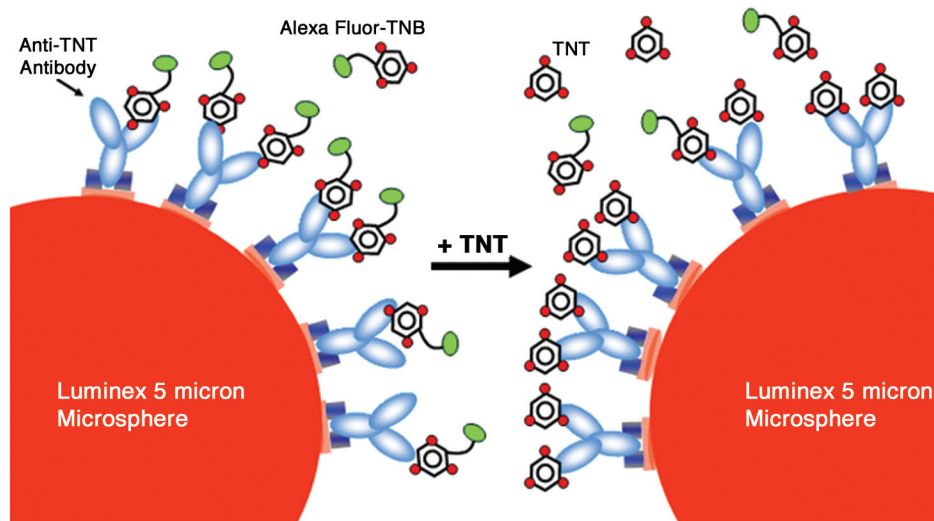


FIGURE 10

Fluid-array displacement TNT immunoassay schematic. First, the Luminex beads are saturated with Alexa Fluor-TNB, making them highly fluorescent. When TNT is added, AF-TNB is displaced and the beads become less fluorescent, causing the measured signal to decrease.

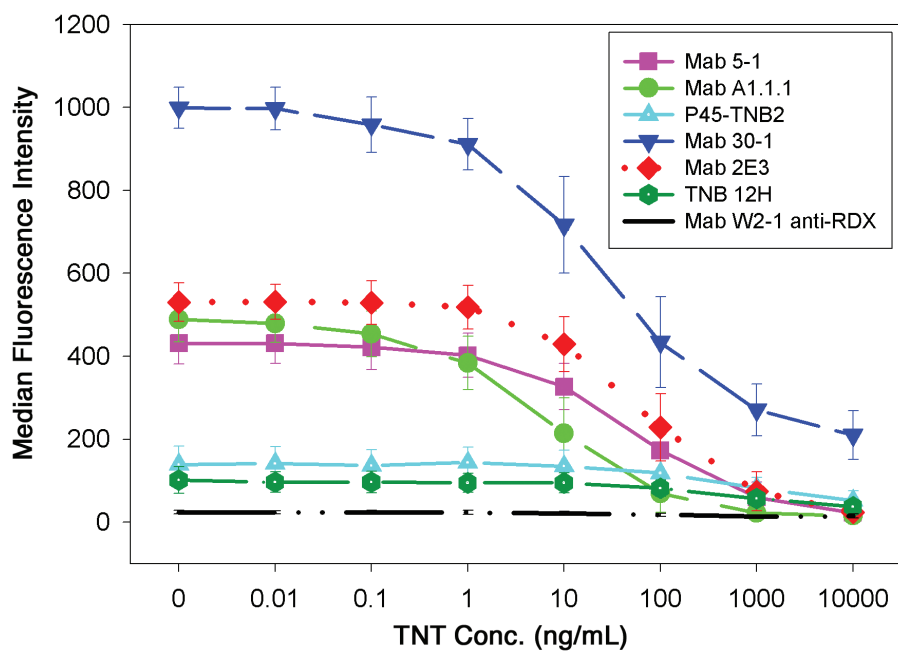


FIGURE 11

This figure shows the median fluorescence value obtained upon measuring each anti-TNT antibody coated Luminex bead set exposed to TNT of various concentrations, which induces displacement of a previously bound AF-TNB tracer molecule.

Table 1 — Cross-Reactivity to Nitro Compounds

Explosive Compound	Anti-TNT Antibodies					
	5-1	A 1.1.1	P45-TNB2	30-1	2E3	TNB12H
TNT	1	1	1	1	1	1
TNB	14	26	1.2	4	17	1.2
Tetryl	0.3	0.1	11.2	0.5	0.3	23
2-A-4,6-DNT	71	91	11	172	108	25
2,4-DNT	108	555	42	625	86	22
RDX	277	3333	10	909	476	111

Cross-reactivity factors of the anti-TNT antibodies to nitroamines and nitroaromatics. Values were calculated by taking the concentration of each compound required to cause 50% dissociation of the AF-TNB, then dividing by concentration of TNT to achieve 50% dissociation. Cross-reactivity factors greater than one indicate that the compound is less effective than TNT at displacing AF-TNB from the antibody-coated beads.

Table 2 — TNT Quantitation in Soil Samples

Soil Sample	Antibody-Coated Bead Type				Avg	HPLC
	5-1	A1.1.1	30-1	2E3		
G18-L1-A	28.9	24.4	19.4	16.1	22.2	16.2
G16-L2-A	17.9	9.4	7.0	10.4	11.2	6.2
G18-L3-A	7.6	6.9	3.9	5.2	5.9	1.7
G51-L1-A	3.4	4.1	1.1	2.7	2.8	1.5
G55-X-A	350	261	82	291	246	238

The concentration of TNT in each of the soil samples was determined in the displacement assay using several monoclonal antibodies. TNT concentrations in the same samples were also determined using the HPLC standard method employed by the EPA.

simultaneously monitor for a range of environmental contaminants in a rapid, high-throughput manner may make multiplexed fluid-array displacement immunoassays the analytical method of choice in the future.

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References

¹ J.P. Whelan, A.W. Kusterbeck, G.A. Wemhoff, R. Bredehorst, and F.S. Ligler, "Continuous-Flow Immunosensor for Detection of Explosives," *Anal. Chem.* **65**, 3561-3565 (1993).

² P.T. Charles, L.C. Shriver-Lake, S.C. Francesconi, A.M. Churilla, J.G. Rangassammy, C.H. Patterson, J.R. Deschamps, and A.W. Kusterbeck, "Characterization and Performance Evaluation of In vivo and In vitro Produced Monoclonal Anti-TNT Antibodies for the Detection of TNT," *J. Immunol. Methods* **284**, 15-26 (2004). ★